IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS:	**HARDIN ET AL.**	§	ART UNIT NO.: **1633**
SERIAL NO.:	**09/901,782**	§	EXAMINER: **SMITH, CL**
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		§	
TITLE: **REAL-TIME SEQUENCE DETERMINATION**		§	
		§	

571-273-8300 FACSIMILE NO.	CERTIFICATE OF MAIL BY FACSIMILE TRANSMISSION	1 February 2007 Date of Deposit
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DECLARATION OF DR. SUSAN H. HARDIN

My name is Dr. Susan H. Hardin, and I am over 18 years of age and am a named inventor on this application. I am submitting this declaration to provide documentary support to antedate the cited Korlach et al. reference as it relates to beta or gamma labeled nucleotides.

I declare as follows:

I have thoroughly reviewed the cited Korlach et al. application, its parent application filed on 17 May 2000, and the 1999 provisional application from which it claims priority.

A thorough review of the Korlach et al. 1999 provisional application shows that the provisional application contains no disclosure of beta or gamma labeled nucleotides.

I have been advise by patent counsel that because the Korlach et al. 1999 provisional application does not disclose beta or gamma labeled nucleotides, documents dated prior to 17 May 2000, the filing date of the non-provisional Korlach et al. patent application, are all that is required to antedate the Korlach et al. reference as it relates to beta or gamma labeled nucleotides.

Attached are relevant sections of a document generated by Applicants prior to 17 May 2000 that disclosed the use of nucleotides labeled on the pyrophosphate, specifically the gamma or terminal phosphate, in sequencing strategies.

Upon instructions from patent counsel, the actual document, its source and the actual date of its creation are not included, such information is being withheld because of potential interference proceedings involving a patent application of Applicants and one or more issued Korlach et al. patents.

I hereby declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 1 February 2007

Respectfully submitted,

Susan H. Hardin

A. TITLE: Real-time Sequence Determination

D. Statement of the Approach

We have assembled a research team with complementary areas of expertise in 1) Molecular Biology, Biochemistry, and Chemistry; 2) Computer Science; and 3) Chemical and Mechanical Engineering:

Dr. Susan Hardin provides expertise in molecular biology, DNA replication, and DNA sequencing. Dr. Hardin's group will identify the optimal enzyme to use for our studies. They will also genetically modify the gene encoding DNA polymerase, sequence the resulting polymerase clones, and assay enzyme activity.

Dr. Shiao-Chun (David) Tu provides expertise in energy transfer reactions, as well as protein purification and enzymology. Dr. Tu's group will identify optimal dyes for both enzyme and dNTP fluorescent-tagging experiments. They will also be responsible for fluorescently modifying, purifying, and characterizing engineered polymerase.

Dr. Xiaolian Gao provides expertise in chemical synthesis of unusual deoxynucleoside triphosphates. Dr. Gao's group will design, synthesize, and **purify tagged dNTPs (base, sugar, or phosphate labeled)**.

Dr. James Briggs provides computational expertise. Dr. Briggs' effort includes identification of candidate amino acids for targeted mutagenesis of the polymerase via modeling of the complex between the (labeled) dNTP and the (labeled) protein. The efficiency of the fluorescence resonance energy transfer (FRET) will be predicted. Dr. Briggs' group will also work closely with Dr. Hardin's group to create the base identification software.

Dr. Richard Willson provides expertise in fluorescence, as well as chemical- and instrument- engineering. Dr. Willson's group will be responsible for optimizing larger-scale expression and purification of the polymerase. They will also identify and develop equipment that will meet our needs for both development and single-molecule detection stages of the project.

F. THE IDEA

A brief overview of the proposed single-molecule DNA sequencing process follows: We envision placing a single tag on the polymerase and a unique tag on each dNTP. As a tagged dNTP is incorporated into the DNA polymer, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and/or strength provide signature for base identity). Tagged dNTPs will be identified that do not interfere with Watson-Crick base pairing or significantly impact polymerase incorporation. Initially, we will focus on dyes used to fluorescently label ddNTPs for automated DNA sequencing, since they are incorporated in a template-directed manner by the polymerase. **Additionally, we will determine whether dNTPs containing tags attached to the terminal (gamma) phosphate are directly detected upon incorporation (four color, base-specific phosphate cleavage stimulates detector). An advantage of this latter approach is that the nascent DNA strand will not contain fluorescent bases and, therefore, should produce minimal enzyme distortion and background fluorescence.** The fluorescent signals produced upon incorporation will be detected and analyzed to determine DNA base sequence.

Our Approach

A brief overview of the proposed single-molecule DNA sequencing process follows: In the first approach, we envision placing a fluorescence donor on the polymerase (i.e. fluorescein or fluorescein-type molecule) and a fluorescence acceptor with a unique fluorescent tag color on each dNTP (i.e. d-rhodamine or similar molecule). As incoming fluorescently-tagged dNTP is bound by the polymerase for DNA elongation, a characteristic fluorescent signal is emitted that indicates base identity (emission wavelength and intensity provide signature for base identity). Fluorescently-tagged dNTPs will be identified that do not interfere with Watson-Crick base pairing or significantly impact polymerase incorporation. Initially, we will focus on dyes used to fluorescently label ddNTPs

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Preliminary results

The Cartesian coordinates for GTP were obtained and used in a manual docking experiment to generate a complex between GTP and a DNA polymerase I. The X-ray structure for the polymerase was that from *B. stearothermophilus* with a DNA primer template bound (Kiefer *et al.*, 1998; pdb code: 2bdp). The GTP was manually placed in the proposed dNTP binding site according to the procedure described in Kiefer *et al.*, 1998 (see Figure 4 and associated description in the text). The most relevant points are that at least one oxygen from each phosphate in GTP was within ca. 3.0Å of the observed Mg^{2+} ion and that the base partially stacks with the base at the end of the primer strand.

Figure: DNA polymerase I from *B. stearothermophilus* (Kiefer *et al.*, 1998; pdb code: 2bdp) co-crystallized with DNA template primer and with a manually docked GTP molecule. The protein is represented by a blue solid tube and the DNA by a yellow backbone trace and bonds. The GTP is near the center of each image represented in ball and stick. The rust sphere near the phosphates in GTP is the Mg^{2+} ion bound to the highly conserved Asp653 and Asp830 protein sidechains. In the right most image, the molecular surface of the protein is displayed along with DNA and GTP. GTP is atom colored and Mg^{2+} is rendered in black.

It is clear from the initial molecular modeling studies that the dNTP can be labeled on sites other than the traditional 7-position on purines and the 5-position on pyrimidines. One of the ideas presented in this proposal is to put the fluorescent tag on the γ-phosphate such that, upon base incorporation, the tagged PPi will diffuse away from the protein (i.e. FRET will cease). According to our preliminary modeling studies, and the GTP/protein complex model presented in Figure 4 of Kiefer *et al.*, 1998, there appears to be sufficient room for a tag on the g-phosphate, without inhibiting incorporation.

Selection of site in dNTP to accept fluorescent tag

Molecular docking simulations will be carried out to predict the docked orientation of the natural and fluorescently labeled dNTPs using the AutoDock computer program (Morris *et al.*, 1998;

Soares *et al.*, 1999). Conformational flexibility will be permitted during the docking simulations making use of an efficient Lamarckian Genetic algorithm implemented in the AutoDock program. A subset of protein sidechains can also be allowed to move to accommodate the dNTP as it docks. The best docked configurations will be energy minimized in the presence of a solvent environment. Experimental data are available which identify amino acids in the polymerase active site that are involved in catalysis and in contact with the template/primer DNA strands or the dNTP to be incorporated. The docking studies will help us to support which sites in the dNTP can be labeled and to predict the FRET efficiency that we might expect.

POLYMERASE ACTIVITY ASSAYS USING A FLUORESCENTLY-TAGGED ENZYME AND/OR dNTP(S)

We will monitor the activity of polymerase variants throughout enzyme development. Enzyme activity will be assayed after a candidate amino acid is mutated to cysteine and following fluorescent tagging of that cysteine. A similar assay will be used to monitor the ability of a polymerase or a polymerase variant to incorporate fluorescently-tagged dNTPs. Since the enzyme's amino acid sequence will be altered, we will determine whether enzyme characteristics are altered (thermostability, fidelity, polymerization rate, affinity for modified versus natural bases). Similar procedures will be used to identify the optimal reaction buffer.

FLUORESCENT TAG CHOICE AND ADDITION

Approach 1

The following principles will be guiding our search for appropriate fluorescence dyes for this work. In one approach, a fluorescence donor will be attached to the polymerase and four unique fluorescence acceptors will each be attached to a different dNTP. The absorption spectra of the donor and acceptor fluorophores should be sufficiently distinct to allow exclusive (preferably) or preferential excitation of the fluorescence donor attached on the polymerase at a chosen wavelength. The emission of the fluorescence donor should have significant overlap with the absorption spectra of the fluorescence acceptors. The four fluorescence acceptors, in the dNTP-attached forms, should each have a unique fluorescence emission distinguishable from that of the other three.

Several sites on dNTPs will be explored for the attachment of the fluorescence acceptors. **The initial efforts will be directed to the tagging of the terminal phosphate of dNTP. This approach has a unique advantage. When the incoming, tagged dNTP is bound to the active site of the polymerase, significant FRET from the donor on the polymerase to the acceptor on the dNTP is expected to occur. The unique fluorescence of the acceptor then enables the determination of the identity of the dNTP. Once the tagged dNTP is processed for covalent attachment to the nascent DNA chain, the fluorescence acceptor remains attached to the pyrophosphate and will be released to the medium. In fact, the growing nascent DNA chain will contain only the normal dNMP building units and no fluorescence acceptor molecules at all. In essence, FRET will only occur between the donor on the polymerase and incoming acceptor-labeled dNTP, one at a time. This approach is better than the alternative attachment of the acceptor to any site within the dNMP moiety of the initial dNTPs. In this latter case, the nascent DNA chain will contain multiple molecules of the fluorescence acceptors. Interference with the polymerase reaction and FRET measurements could occur.**

CHEMICAL MODIFICATION OF NUCLEOTIDES FOR DNA POLYMERASE REACTIONS

Significance

A high level incorporation of dNTPs is crucial for the success of the proposed project. It is, thus, requiring intense chemistry effort in synthesis of modified dNTPs to engineer features that would permit high fidelity enzymatic synthesis and sensitive detection. Presently, a number of dye-labeled dNTPs are available from commercial resources. However, the protein-DNA complex system used in our method imposes demands that are more stringent, such as null background

signals with minimal interference in multi-fluorophore systems. These requirements cannot be completely satisfied by commercial products.

Research Plan

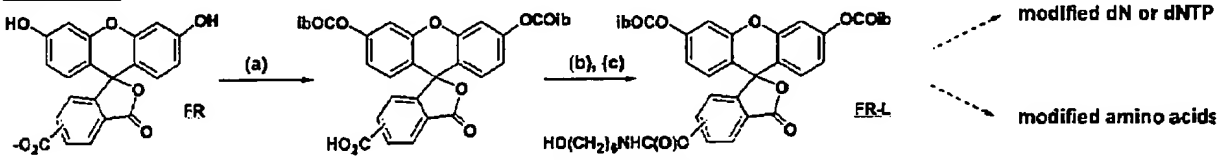
The proposed synthesis will be based on the nucleoside/nucleotide chemistry developed in this laboratory as part of the antisense oligonucleotide (AON) project. In the AON project, our interests are to understand the correlation of chemical structure modifications with AON binding affinity and specificity in target sequences. Using chemistry and high resolution NMR in combination, our laboratory has characterized a series of AONs (Gao *et al.*, 1992; Rice and Gao, 1997; Cross *et al.*, 1997; Gao *et al.*, 1997; Yang *et al.*, 1999). The chemistry of modified nucleotides used in the AON project is directly applicable for the proposed synthesis. This background would permit us proceed rapidly to achieve synthesis of modified dNTPs.

In the proposed project, we will work closely with Dr. Tu in selection of molecules for signal detection. We initially choose to use the popular fluorescing molecules, such as rhodamine and fluorescein derivatives, and utilize the fluorescence resonance energy transfer (FRET) phenomenon (Foster, 1965; Ju *et al.*, 1995; Lee et al, 1997; Furey *et al.*, 1998). Alternatively, chromophore interactions as in a fluorophore-quencer pair (Tyagi and Kramer, 1996; Tyagi *et al.*, 1998; Fang *et al.*, 1999) or a fluorophore-excimer pair (Yamana *et al.*, 1997; Tong *et al.*, 1995; Paris *et al.*, 1998; Lewis *et al.*, 1997) may be considered. Together, these molecules are called tags. In these designs, we would need to place a tag on the polymerase and its energy partner tag on the dNTP. The choice of fluorophore is a function of not only its enzyme compatibility, but also its spectral and photophysical properties. For instance, it is critical that the acceptor fluorophore does not have absorption (*i.e.*, at least less than 1/1000) at the excitation wavelength of the donor fluorophore, and that the donor fluorophore does not have emission at the detection wavelength of the acceptor fluorophore. These spectral characteristics may be attenuated by chemical modifications of the fluorophore ring systems. Absorbance and emission spectra of the modified fluorescing molecules will be examined to satisfy the requirements discussed above.

In the following, we provide reaction routes that serve as examples for the proposed synthesis. These synthesis reactions have been used in our on-going projects in the AON area and DNA microarrays and demonstrate our current effort and capability for developing the chemistry to meet the demand of the proposed project.

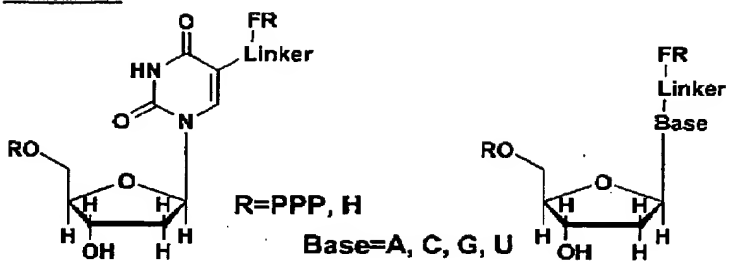
Synthesis of fluorescein derivatives. Fluorescein (FR) molecules will be modified to contain a linker unit. These molecules can be covalent attached to nucleotides (Ward *et al.*, 1987; Engelhardt *et al.*, 1993; Little *et al.*, 2000; Hobbs, 1991) or amino acids. A representative synthesis is shown below (Scheme 1). The product FR-L can be used to attach to nucleotides and amino acids. Other fluorophore molecules may be modified using similar type of chemistry.

Scheme 1



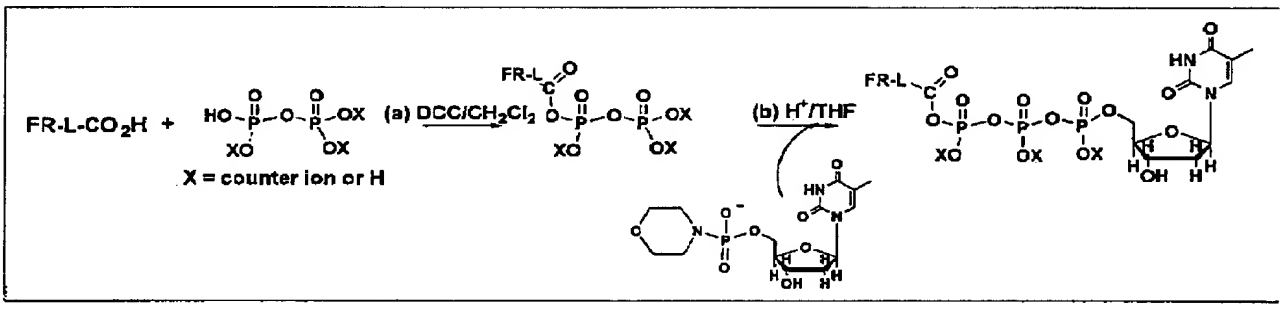
Synthesis of base and sugar modified nucleotide dU. Nucleobases and sugar moieties can be modified with a fluorophore, yet still maintain their enzymatic reaction activity. The modifications are also selected as sites that do not interference with Watson-Crick base pairing. The basic structural scheme for base modification is shown in Scheme 2. Our laboratory routinely prepares nucleotide derivatives in milligram quantities and has procedures for preparation of tagged nucleotides, which are not commercially available.

Scheme 2



Synthesis of modified dNTPs. We hypothesize that polymerase may be able to utilize phosphate-modified dNTPs. If a tagged γ-phosphate ester can be used as a substrate, then the tag will be removed by the enzyme after nucleotide incorporation. Since the replicated DNA will not contain any unnatural bases, polymerase activity is less likely to be affected and extended strands should result. We will synthesize phosphate-modified nucleotides using adapted literature procedures (Bonnaffe *et al.*, 1995). An example for the reaction of γ-phosphate modification is shown in Scheme 3.

Scheme 3



Our synthesis will initially focus on pyrimidine nucleotides and identify suitable tags. Effort will also be made to change the substituents on fluorophore and relevant molecules. These chemical conversions may be necessary for achieving sufficient levels of incorporation by the polymerase. Additionally, it is anticipated that multicolor (or intensity) detection will improve confidence values associated with the base calling algorithm. These compounds will be tested initially in our laboratory and then by the project collaborators. Chemistry will be constantly revised according to input from these laboratories.